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Isolation and characterization of exosomes derived from supernatant of rabbit kidney tubular cells culture

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Abstract

Introduction: Different vesicles are created by the cells into extra-cellular space, of which researchers mostly considered exosomes. In fact, the vesicles are secreted by each cell type and are found in the body fluids like urine, blood and breast milk and consist of a certain ingredient of proteins, RNA, lipids and DNA.
Objectives: The present study attempted to examine the exosomes derived from tubular cells of the rabbit kidney.
Materials and Methods: Exosomes have been derived from rabbit kidney tubular cell line (RK13Cs) supernatant by ultracentrifugation centrifugation treatment techniques. In the next stage, RK13Cs-Exo has been validated by the dimension, morphology and certain bio-markers through the transmission electron microscopy (TEM) and scanning electron microscopy (SEM), Zetasizer nano-analysis, atomic force microscopy (AFM) and western blotting.
Results: The morphology of RK13Cs-Exo under TEM and SEM revealed that they have been vesicles shaped like a sphere ~150 nm. Zetasizer nano also indicated that RK13Cs-Exo size has been nearly 50 to 100 nm. Atomic force microscopy image of RK13Cs-Exo showed distinct spherical particles in size range between 50 and 100 nm, corresponding to the TEM. According to the western blotting outputs, exosome markers CD63 and CD9 have been expressed in the RK13Cs-Exo.
Conclusion: Tubular cells of the rabbit kidney synthesize large amounts of exosomes under critical environmental conditions, which can extract the produced exosomes. According to the characterization of derived exosomes, they are ideal vehicles for drug delivery.

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Introduction

Exosomes are the derivation of the endocytic membrane with the ability to transfer different signals to the recipient cells; therefore, they mediate a new system of the cell-to-cell relationship (1). In fact, the exosomes play a role in various biological procedures like the presentation of antigen, angiogenesis, apoptosis, coagulation and inflammation. These exosomes are capable of activating the signaling paths or delivering the nucleic acids to the distant cells (2). Moreover, the tumor-derived exosomes may augment the cancer expansion, which inhibits the immune responses and transfer of oncogenes from the host cells of the tumor (3). In addition, targeting exosomes is beneficial for increasing the treatment efficiency of antibody. Furthermore, the exosomes that are generated by navigating the endosomal membranes producing the multi-vesicular bodies (MVB) consist of mRNA and miRNAs (4). On the one hand, the protein compound reflects their endocytic source, machinery of the MVB creation, tetraspanins; for example, CD9, CD63, CD82, and CD81, heat shock proteins and the lipid-associated phospholipases and proteins and also their probable contribution to the inter-cellular communication (5).

In fact, exosomes involve significantly in a variety of biological functions like transferring the bio-molecules, including DNA, RNA, enzymes, proteins and lipids as well as in regulating multiple pathophysiological procedures in different illnesses (3). The identification of miRNAs

Key point

In this study, we derived exosomes from rabbit kidney tubular cell line (RK13Cs) supernatant by ultracentrifugation centrifugation treatment techniques. We found tubular cells of the rabbit kidney synthesize large amounts of exosomes under critical environmental conditions, which can extract the produced exosomes. According to the characterization of derived exosomes, they are ideal vehicles for drug delivery.

and mRNAs in the exosomes and documents of their involvement in exosomes in the cell to cell communication indicates one of the novel prominent directions, which is utilization of exosomes as the delivery devices for treatment options (4).

Multiple investigations indicated that the exosomes derived from a variety of cells have various performances as the same the mesenchymal stem cells (MSCs) functions like restoration of the tissue damages, suppression of the inflammatory response and modulation of the immune system (6). However, in comparison to the cells, exosomes have higher stability and greater reserve supply. Moreover, exosome therapy can be a candidate method instead of cell therapy in a variety of diseases (7). Therefore, for examining the exosomes applications in the biomedical field, better understanding of the fundamental molecular systems related to transporting and functioning such vesicles is very necessary. Herein, we investigate the separation and description of the exosome's specifications derived from the rabbit kidney tubular cells.

Objectives

Our research aimed at addressing essential parameters for directly separating the exosomes and implementing the obtained information for presenting a direct, simplified and reliable technique to assure the exosome characteristics.

Materials and Methods

Culture of rabbit kidney tubular cell line (RK13-Cs) in vitro

According to the research design, the rabbit kidney tubular cell line (RK13; NCBI Code: C523) has been purchased from the Pasteur Institute of Iran and then immediately transferred to the Dulbecco modified Eagle's Medium (DMEM) high glucose (Gibco, UK) composing of 2 mM L-glutamine and 100 U penicillin/streptomycin (all from Invitrogen; Waltham: MA) and10% fetal bovine serum (Gibco, UK) and stored in a 37°C incubator and 5% CO_2 for proliferation. Then, we replaced the medium after five days and used Phosphate Buffered Saline (PBS) to wash it in order to remove the non-adherent cells. The cells have been moved by means of 0.25 % trypsin upon reaching~90 % confluence.

Isolation of the kidney tubular cells-derived exosomes

RK13Cs-Exo have been procured and treated according to the previous description by Kosaka et al (8). After the cellular confluence reached 80–90 %; PBS has been used to wash them, then they have been cultured at 37°C and 5 % CO2 in a certain RK13Cs medium without FBS for further 48 hours. Isolation of exosomes has been accomplished over the supernatant of the cell culture at the second passage and 48 hours following the cultivation in the serum-free DMEM. Then, the conditioned medium has been collected and several centrifugation steps have been taken at 300g for 10min, 2000 g for 10 minutes and 10 000 g for 30 minutes at 4°C, causing removal of the cells, big dead cells as well as the debris. When centrifugation has been done, a 0.22 µm filter sterilize Steritop™ (Millipore: USA) has been used to filter the supernatant for eliminating the rest cells and cellular debris. Afterwards, the supernatant has been transported into an Ultra-clear tube (Millipore: USA) and high-speed centrifuged at 60 000 × g at 4°C for 90 minutes to isolate purified exosomes as a final pellet (9). Next, it has been decanted and the RK13Cs-Exo pellet has been re-suspended in 200 µL of PBS.

Determination of exosomal solution protein concentration

In order to estimate the concentration of the isolated exosomes, its protein has been measured by Bradford solution and then its standard diagram has been plotted employing the sequential dilutions of BSA protein at the specified concentration. According to this method, 100 µL of the exosomal solution sample has been added to 5mL of Bradford reagent and the absorbance has been read at 595 nm. Moreover, we independently added 100 µL of the distilled water to 5 mL of Bradford reagent and used it as the blanks. In addition, concentrations of 10 to 50 µg/mL of bovine serum albumin have been made to prepare the standard curve. Then, 100 µL of each standard concentration has been blended with 5 mL of Bradford solution and kept at room temperature for 5 minutes. Finally, their absorbance has been recorded at the desired wavelength.

Evaluation of the exosome's morphology by TEM and SEM

Transmission electron microscopy (TEM) has been used to observe the morphology of the exosomes. At first the exosomal pellet was fixed with 1% glutaraldehyde (Sigma) and then 20 µL of the fixed exosomes have been inserted on a carbon-coated grid and allowed to dry at room temperature for 30 minutes. Then, LEO 906 TEM (Zeiss: Germany) has been employed to analyze the sample, which has been operating at 80 kV following washing the grids two times by PBS for 5 minutes and staining with 1% uranyl acetate for 10 minutes. Afterwards, Digital Micrograph Software (Gatan Inc) has been conducted to record the TEM images with Orius 200 camera (Gatan Inc., Washington: DC). In addition, for the particles surface morphology, we used scanning electron microscopy (SEM) and 1 to 5 µL of the dried sample on the silicon chip after fixing with 2% paraformaldehyde. SEM has been used to capture the images at 30 kV after sputtering gold–palladium.

Determination of the distribution and size of exosomes using dynamic light scattering

The dynamic light scattering (DLS) method has been conducted to analyze the solvent nanoparticle, which is capable of measuring particles in solution quickly, easily

and without sample preparation. For this purpose, the extracted exosomes have been resuspended by PBS to 330 µl. After shaking the solution, Malvern Zetasizer Nano ZS has been conducted to measure the size of the exosomes.

Assay of RK13Cs-Exo by atomic force microscopy

After preparing the exosomal pellet over several rounds of high-speed centrifugation, the pellet has been diluted in 5 cc of deionized water and vortexed. Then, the exosomal solution has been transferred to the south-central laboratory of Ahvaz Jundishapur University of Medical Sciences. Moreover, distribution, size, nanoparticles (NPs) size, characteristics, morphology and surface features of the soluble exosomes have been analyzed by atomic force microscopy (AFM) and finally the two-dimensional images have been analyzed.

Western blotting analysis of RK13Cs-Exo surface markers

According to the studies, the presence of the specific surface markers CD9 and CD63 proved RK13Cs-Exo identity (10). Briefly, exosomes have been lysed with RIPA/PI buffer (Santa Cruz Biotechnology; Dallas, TX, USA) and the level of protein has been estimated by Bradford protein assay. Next, RK13Cs-Exo protein has been loaded and solved in 12% sodium dodecyl sulfate/poly-acrylamide gel electrophoresis. In addition, the protein samples have been run at 120 V for 45 minutes and transported in the membrane of polyvinylidene difluoride (Millipore: USA) at 100 mA for 1.5 hours. Moreover, we exposed the membranes to the primary rabbit polyclonal anti-CD9 (1:1000) and anti-CD63 (1:1000) (Abcam, UK) in order to test the presence of CD63 and CD9. Of course, the membranes have been washed three times in 1×Tris-HCl buffered saline Tween (TBS and 0.1% Tween 20; TBST) for five minutes. Then, incubation has been done in TBST with the horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Abcam) for one hour. Finally, the improved chemiluminescence (Thermo Fisher: USA) has been used to detect the proteins, then an Image Quant LAS 4000 mini biomolecular imager (Bio-Rad, USA) has been used to image them.

Results

RK13Cs phenotype

Rabbit Kidney tubular cell lines have been observed in the cell-culture flask approximately every day following the early plating. Then, RK13Cs have been approved by means of a light microscopy for verification of an epithelial-like morphology (Figure 1). Epithelial-like cells showed high ability of rapid growth and approximated 95% confluence after 3-4 days (Figure 1).

Characterization of RK13Cs-Exo

The isolating procedure of the exosome has been conducted to confirm the size distribution in the exosome solution using DLS that has been consistent with SEM and TEM.

Verification of quality and structure of exosomes by TEM

According to the research design, the exosomes generated by the RK13Cs have been separated from the culture medium by differential centrifugation and described by TEM (Figure 2). Moreover, ultra-structural analysis of the exosome pellets using the TEM represented considerable reinforcement of the typical spherical-shaped exosomes with diameter of 50–150 nm (Figure 2). In addition, TEM supported the exosomes average diameter (≤150 nm) with the maintained intact round particles morphology. Furthermore, results showed that the exosomes had a membrane (Figure 2).

Determination of the exosome's concentration by Bradford method

It is notable that a standard protein concentration curve has been plotted by using Bradford solution and sequential dilution of BSA with the specified concentration. Then, the concentration of the isolated exosome protein has been calculated by the line diagram formulas, which have been 2512 µg/mL. Of course, this test aimed to determine the protein in the sample.

Analysis of exosomes with SEM

According to the research design, SEM has been used to evaluate the exosomes size and shape. Moreover, small vesicles with round morphology have been observed in the micrographs obtained from the electron microscopy. In addition, the size of the vesicles has been determined to be 70–160 nm based on the smallest and largest vesicles seen in multiple images. However, the majority of the vesicles have been smaller than 150 nm in size (Figure 3).

Dynamic light scattering analysis

Measuring the exosome size by dynamic light scattering

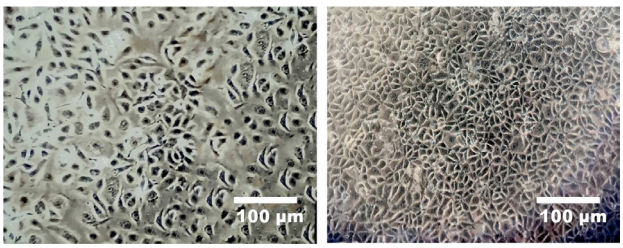

Figure 1. The morphology and growth of RK13Cs after 2 days (left panel) and 4 days (right panel), (100×). Scale bar =100 µm

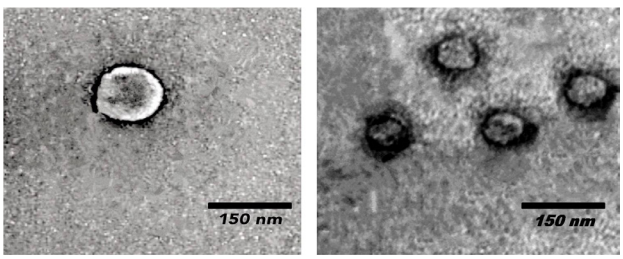

Figure 2. TEM confirmed the average diameters of exosomes to be ≤150 nm with preserved intact spherical morphology.

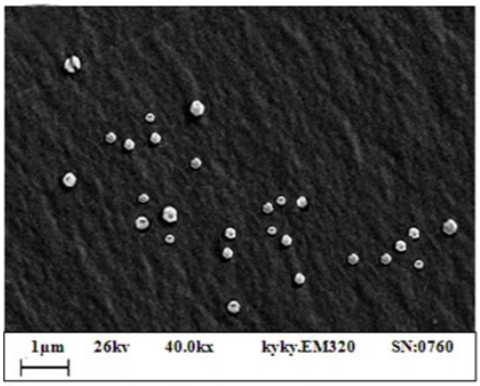

Figure 3. Micrographs of scanning electron microscopy showed spheroid shaped vesicles at the diameter of about 70 to 160 nm.

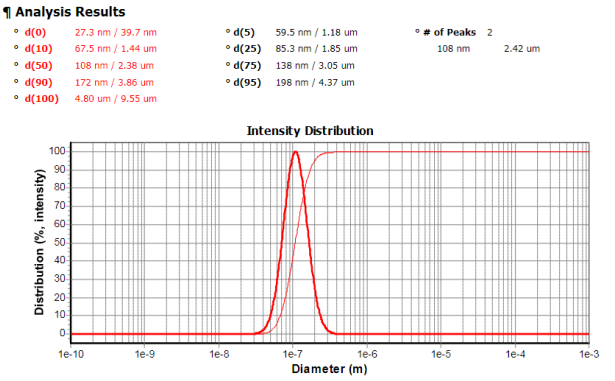

Figure 4. Results of DLS showed that almost 50% of the solution ingredients presented an average diameter of 108 nm.

(DLS) reflected a bell-shaped size distribution with a peak of about 108 nm. It means that nearly 50% of the solution constituents showed the average diameter of 108 nm (Figure 4).

Western blotting analysis

Researchers utilized the proteins on the outside layer of the exosomes membranes as the markers for their identification. Notably, the most common markers in this field are the tetraspanin family markers identified by western blotting to confirm the nature of the exosomes. In addition, this study evaluated the expression levels of CD9 and CD63 by the western blotting to determine the nature of the isolated exosomes. As can be seen in Figure 5, the results of the western blotting showed the expression of the exosomes markers like CD63 and CD9 in RK13Cs-Exo (Figure 5).

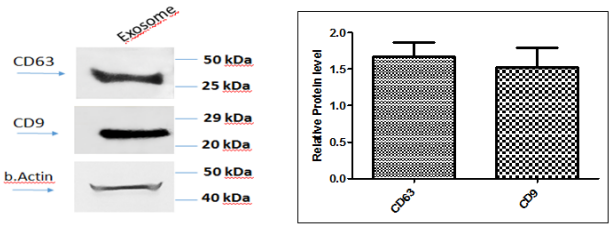

Figure 5. The exosomes markers CD9 and CD63 were expressed inRK13Cs-Exo .

AFM analysis

The AFM obtained from the exosomes showed that the NPs are symmetric, spherical and without aggregation. Moreover, size of the NPs ranged 60 to 150 nm (Figure 6).

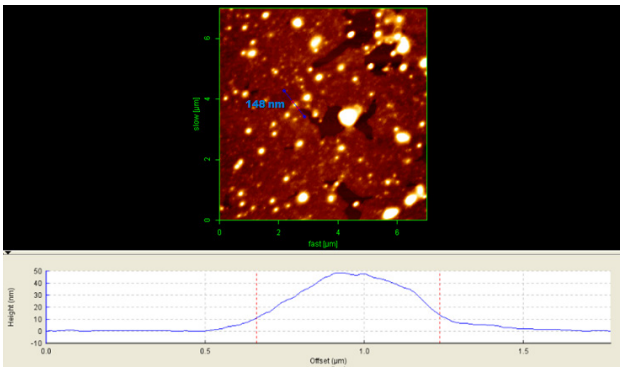

Figure 6. AFM image of RK13Cs-Exo showed distinct spherical particles in size range between 60 and 150 nm

Discussion

This study isolated exosomes from RK13Cs and characterized them. Nowadays, sequential and differential centrifugations are a general strategy to isolate the exosomes. However, other approaches, including the inclusion of magnetic beads consisting of the specific antibody in the proteins of the surface of the exosomes in downstream isolation of the pre-enrichment protocols have substantially resolved the problem of the reduced purity (11). In addition, various studies compared two methods of ultracentrifugation with the density gradient centrifuge method hence, results showed higher efficiency of the density gradient centrifugation, while ultracentrifugation is a cheaper method. Therefore, we used ultracentrifugation (60 000 g) to isolate the exosomes derived from the renal tubular cells present in the cell soup and characterized them by electron microscopy. Moreover, the study conducted the dynamic light scattering method and showed that approximately 50% of the vesicles isolated by ultracentrifugation had an average diameter between 108 nm. Furthermore, comparison of the two separation methods with ultracentrifugation (i.e., 100 000 g and 60 000 g) results from other studies indicated that the exosomal particles separated by 100 000 g are purer; however, particles larger than exosomes such as micro-vesicles may be seen in the extraction with 60 000 g (12).

Based on the normal and pathological conditions, the cells in the body release their own vesicles called vesicles the microvasculature and exosomes release, which have attracted the attention of many researchers in recent years. These vesicles contain membrane protein and phospholipid compounds, including receptors and cell surface molecules. There are also many molecules within the cytosol inside these vesicles. Therefore, exosomal

therapy could be used as an alternative cell therapy due to the side effects of the direct injection of stem cells (13). According to the investigations, it has been found that exosomes contribute importantly to the promotion of angiogenesis. In addition, in-vitro and in-vivo examination with the exosomes derived from human CD34+ cells reflected an angiogenic activity. Moreover, it is possible to induce benefits of the CD34+ cell treatment on the functional recovery following the ischemic injuries basically through the exosome-mediated transfer of the angiogenic variables to the neighboring cells (14).

Moreover, studies showed that in the rat model of the severe kidney injuries induced by cisplatin, those rats given treatment with the human umbilical cord MSC-derived exosomes demonstrated lower severe kidney injury and lower levels of creatinine and nitrogen of the blood urea, necrosis, apoptosis and oxidative stress. In addition, the exosomes from the MSCs could decline the cell mortality signaling by Bax, enhance the survival signaling by Bcl-2 and finally modulate apoptosis and rapid growth in the kidney during injuries. Such a result represents treatment capacity of the MSC-derived exosomes (15). Furthermore, previous authors reported miR-146b expression for inhibition of the glioma growth by the marrow's stromal cell-derived exosomes. Therefore, the exosomes function as the biological delivery vehicles because of the lack of the immune rejection and the risk-free tumor formation (16). The renal ischemia and reperfusion have been proved to induce renal microvascular endothelium activation and elevate chemokines expression, which always mediate monocytes adhesion and migration into the injured tissue (17). One of the most important chemokines contributing to the pro-inflammatory monocytes recruitment, CCL2 (C-C motif chemokine receptors and its major receptor CCR2 are the key factors, regulating the tubular injury after renal I/R in the mice models (18). In addition, it has been found that CCR2 is enriched in the MSC-derived exosomes and these CCR2 positive exosomes strongly bind to extracellular CCL2 and reduce its concentration. Li et al, therefore, suggested that the CCR2 positive exosomes play a role as the decoys to suppress CCL2 function and then inhibit recruiting and activating the peripheral monocytes/macrophages (19).

The blood–brain barrier (BBB) is responsible for protecting the brain against hazardous chemicals. Moreover, BBB challenges the drugs to reach the targeted cells. In fact, developing the vehicles for the brain drug delivery could be strongly overshadowed by BBB hurdles. In fact, researchers provided two possible paths for entering the agents into the brain through the nasal cavity. 1) A systemic pathway, through which the medicine scan be adsorbed from the nasal cavity into the systemic circulation by means of a rich vasculature of the respiratory system circulation and thus reach the brain by passing the BBB. 2) Olfactory region located on the top of the nasal cavity creates an olfactory pathway, through which the NPs are immediately delivered to the brain tissues and bypass the BBB (20). It is notable that the drug delivery through BBB is a highly hopeful utilization of nanotechnology in the clinical neuro-science. In this regard, one of the studies demonstrated that intra-nasal administrations of exosomes cause largely effective delivery of anti-signal and curcumin transducer and activator of transcription 3-inhibitor into the brain. Moreover, it has been found that the exosomes injected into the blood function as the "drugs vehicles" and are able to pass the normally impermeable BBB to the brain where their presence is crucial (21). Ultimately, the exosomes potential for transferring to the brain, their non-cytotoxicity as well as efficient delivery agents have opened encouraging windows of more efficient therapeutic options for diseases such as Parkinson's and Alzheimer's diseases.

Conclusion

Studies showed that exosomes consist of proteins and lipid determinants, allowing them to have interactions with the targeted cells. Altogether, the exosomes are capable of signaling the targeted cells at the cell periphery or giving data to cytosol and translation machinery and probably to the nucleus. However, if we want to potentially utilize the exosomes as the treatment vehicles, this is dependent on the reality that secretion and reuptake of the exosomes can move the molecules between the cells (22) that are procedures, which may be broken up or changed by designs. However, it is possible that this design is extended beyond its present main emphasis on the oncology and immune disease for achieving hopeful methods for treating diseases.

Limitations of the study

During the research, we encountered some problems such as inconsistencies in implementation and time constraints.

Authors' contribution

FF, MO and EM contributed to conception and design. FF and EM contributed to all experimental work, data and statistical analysis and interpretation of data, methodology, investigation and cell culture work. MO writing and original draft preparation. FF drafted the manuscript, which was revised by MO and EM. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Ethical issues

Ethical considerations the institutional ethical committee of Ahvaz Jundishapur University of Medical Sciences approved all study protocols (Ethical code# IR.AJUMS.ABHC.REC.1398.008) study was extracted from the Ph.D., thesis of Fereshtesadat Fakhredini at this university. Additionally, ethical issues (including plagiarism, data fabrication and double publication) have been completely observed by the authors.

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